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Update on Electrophysiology

Patch-Clamping and Other Molecular Approaches for the Study of Plasma Membrane Transporters Demystified

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As the boundary between the inside and the outside of the cell, the plasma membrane is one of the most important structures that cells use to regulate their internal composition and activities. Proteins embedded in the plasma membrane control which solutes are accumulated and which are excluded; their activity creates gradients of solutes across the membrane. This process is important for many aspects of plant growth and development, including cell expansion, mineral nutrition, long-distance transport of assimilated carbon and nitrogen, and cellular and whole-plant responses to environmental signals.

Electrophysiology is the study of the transport of charged solutes across membranes and the proteins that carry out this transport. Electrophysiological studies have been performed on plants for at least 50 years. In the last 15 years the highest-resolution electrophysiological method, the patch-clamp technique, has been used extensively in plants. As a result, many plasma membrane transport activities, especially ion channel activities, have been characterized in detail. These activities can now be linked to structural information of proteins and, as a result, we can begin to understand the mechanisms of transport at the molecular level.

Neither traditional genetic approaches nor library screening using heterologous probes has been useful for obtaining transport protein genes. A unique set of molecular tools, including expression cloning in yeast, heterologous expression in *Xenopus laevis* oocytes, reverse genetics to obtain knockout mutants, and high-resolution electrophysiological assays, is being used to begin to create a full description of transport protein structure, function, expression pattern, targeting, and regulation.

This *Update* provides a practical guide for nonelectrophysiologists to understand the results from patch-clamp experiments. This is important because the patch-clamp technique will have additional applications in the future as knockout mutants and heterologous expression become more common. Furthermore, patch-clamp results are difficult to understand for many scientists who have not had personal experience with the technique. The current molecular approaches mentioned above, which are aiding in the rapid progression of the cloning of cDNAs of transport proteins, are also presented here to provide a glimpse into the cutting edge of electrophysiology, where mutant transporters are selected for desired characteristics on a functional basis and where knockout/replacement studies will finally be possible in plants.

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OVERVIEW OF ION AND SOLUTE TRANSPORT AT THE PLASMA MEMBRANE

Some ions and solutes, such as H^+, Na^+, and Ca^{2+}, are actively excluded from cells; others, such as K^+, NH_4^+, NO_3^-, Cl^-, SO_4^{2-}, PO_4^{3-}, sugars, and amino acids, are actively accumulated. The transport of each of these ions or solutes involves specific transport proteins in the plasma membrane. Three general classes of transporters will be discussed in this *Update*: pumps, cotransporters, and channels. Pumps such as H^+-ATPase couple chemical energy (e.g. ATP hydrolysis) to transmembrane ion movement. Cotransporters such as amino acid or Suc transporters couple the transmembrane movement of two different solutes or ions. Channels permit selective diffusion of ions or solutes across the membrane. This classification is useful for the discussion of transport mechanisms but should otherwise be used cautiously since it is possible for individual transport proteins to function in more than one mode.

One fundamental characteristic of plasma membrane transport in plants is that protons are actively pumped out of the cell. This is accomplished by plasma membrane H^+-ATPases that hydrolyze ATP in the cytosol and utilize that energy to pump H^+ out of the cell (for review, see Sussman [1994]). This creates the pH gradient (outside acidic) and the voltage difference or membrane potential (inside negative) that are the primary forms of stored energy across the membrane and are utilized for the accumulation or exclusion of other solutes via cotransporters and channels. The normal pH gradient across the plasma membrane is approximately 1.5 to 2 pH units. The membrane potential varies between different types of cells, but is usually in the range of −100 to −150 mV.

The membrane potential is controlled predominantly by the balance between H^+ extrusion by ATPases and the activity of K^+ channels. The negative potential activates inward K^+ channels and drives K^+ influx through these selective pores (for review, see Schroeder et al. [1994]). This situation changes dramatically when cells are stimulated by factors such as hormones, elicitors, and light, in which up- or down-regulation of H^+-ATPases (Kinoshita et al.,

1995) or the activation of other ion channels (Cho and Spalding, 1996; Keller and VanVolkenburgh, 1996; Pei et al., 1997) can control the membrane potential during signal transduction.

The energetics of the uptake of cations such as K^+ into cells via ion channels are described by the Nernst equation (Hille, 1992), where for each −59 mV of membrane potential, K^+ can be accumulated against an additional 10-fold concentration gradient. Therefore, at a resting potential of −150 mV, a maximum K^+ concentration gradient of 385-fold can be supported. Because of the negative membrane potential, only cations can be accumulated via channel-mediated transport. The active uptake of anions and uncharged solutes occurs via cotransport mechanisms. For example, NO_3^- is accumulated through the activity of an H^+-coupled cotransporter, CHL1 (Tsay et al., 1993). In fact, anion channels serve the opposite purpose: they allow anion efflux during signal transduction (for review, see Ward et al. [1995]).

UNDERSTANDING THE RESULTS OF PATCH-CLAMP EXPERIMENTS

In the early 1980s membrane biophysics was revolutionized by the introduction of the patch-clamp technique (Hamill et al., 1981). The most important innovation was the use of small pipette tips to apply suction, which formed very high-resistance seals between the glass pipettes and cell membranes. This meant that low-noise recordings of small currents (as small as single ion channel currents) could be made. Almost immediately, the first applications in plant electrophysiology were reported (Moran et al., 1984; Schroeder et al., 1984). Since that time many different ion channels in the plasma membranes and intracellular membranes of different kinds of plant cells have been characterized. However, there are several difficulties specific to plants that prohibit the general application of the patch-clamp technique to all types of plant cells.

Plant cells are surrounded by a cell wall that must be removed before patch-clamp experiments can be performed. Therefore, methods for producing suitable protoplasts must first be developed, and methods for identifying protoplasts from a particular type of cell must be available. Molecular approaches, such as ectopic expression, are expected to circumvent these problems, allowing characterization of previously inaccessible transport proteins, as discussed later in this *Update*. In addition, the technique of laser microsurgery has been applied successfully to remove sections of plant cell walls to expose the plasma membrane so that patch-clamp recordings can be made (Henriksen et al., 1996).

For the electrophysiologist, there is abundant introductory information on the patch-clamp technique (Hamill et al., 1981; Hedrich and Schroeder, 1989; Hille, 1992). However, for other scientists and for biology students who wish to understand the results, possibilities, and limitations of patch-clamp experiments, the field sometimes appears shrouded in strange terminology. Since patch-clamp experiments will have even more utility in combination with other molecular approaches and will be more predominant in the future, this *Update* is designed to provide nonelectrophysiologists with enough information to interpret results from patch-clamp experiments.

Analysis of Whole-Cell Currents

One of the typical configurations for a patch-clamp experiment is the "whole-cell mode" (definitions for patch-clamp terminology are given in Table I), which is described in detail elsewhere (Hamill et al., 1981; Hille, 1992). Once a high-resistance seal or "giga seal" is made, the patch of membrane at the tip of the pipette is ruptured so that the pipette solution equilibrates with the cytosol. This situation is perfect for recording currents across the entire plasma membrane of a single cell. One important characteristic of the whole-cell mode is that the composition of the solution on each side of the membrane is known, and therefore determination of which ions are going through channels and other transporters is possible, as discussed later in this *Update*. It should be noted that small solutes diffuse from the patch pipette quickly and equilibrate with the cytoplasm within a few minutes, whereas larger solutes such as proteins may require prohibitively long periods (up to hours) for loading into the cytosol (for details, see Pusch and Neher [1988]).

Experiments are usually performed using a voltage clamp, in which the membrane potential (voltage) is clamped (set or controlled) and the current is measured. An example of voltage-clamp data for a whole cell is presented in Figure 1. In this experiment the membrane potential was held at −60 mV (less negative than a normal resting potential) and was then stepped to progressively more negative potentials. In this example, the membrane potential was first stepped to −120 mV. In Figure 1A the effect on currents across the plasma membrane can be observed. The first important observation is that the direction of the elicited currents is downward; this is strictly a convention and indicates that the currents are inward (either carried by cations moving into the cell, anions moving out of the cell, or both).

The next important observation is that the currents change with time. The ion channels responsible for these currents activate relatively slowly in response to changes in membrane potential, requiring about 1 s for full activation. By comparison, fast channels can activate within 0.1 ms. As shown in Figure 1, after 1.8 s at −120 mV, the membrane potential was then returned to the holding potential of −60 mV, where the channels deactivate. Therefore, these channels are voltage dependent: their activity changes with the membrane potential.

Figure 1A is not the way whole-cell currents are typically presented, but shows the time course for this experiment. The same data are superimposed in a typical format in Figure 1B. It is more apparent in Figure 1B that the currents are composed of two components. The instantaneous downward current, recorded immediately after the membrane potential was stepped to more negative potentials, is a leak current (indicated by arrows in Fig. 1B). This leak current is the sum of all conductances other than the time-dependent current that is the object of this experiment. The

Table I. *Glossary of patch-clamp terminology*

activation the conversion of a channel into a functional state, i.e. the activation of voltage-dependent channels depends on membrane potential, the opposite is deactivation (see also **inactivation**).

bath solution the extracellular solution (in whole-cell mode) and the site of the reference electrode.

conductance the ability of current to move across the membrane; the inverse of resistance, the units are siemens (S), which are equal to 1 Amp $Volt^{-1}$, and typical ion channels have a conductance of 1–150 pS (1–150 $\times$ 10^{-12} Amp $Volt^{-1}$).

gating the opening and closing of a channel pore.

giga seal a high-resistance seal between the patch pipette and the membrane. The name is derived from the typically 5 to 15 GΩ seal that is possible with patch pipettes.

holding potential the set membrane potential between voltage changes in voltage-clamp experiments.

hyperpolarized describes a membrane potential that is more negative than a resting potential; the opposite of depolarized.

inactivation the time-dependent conversion of a channel to a nonfunctional state under conditions that also promote channel activation.

inward current a positive charge movement into the cell, i.e. cations moving into the cell or anions moving out of the cell.

leak any passive conductance that is not due to the channel or transporter under investigation.

liquid junction potential an error in setting the membrane potential in voltage-clamp experiments due to different mobilities of anions and cations in either bath or pipette solutions.

membrane potential the potential difference or voltage difference across the membrane.

patch the piece of membrane sealed to a patch pipette and covering the pipette tip.

patch pipette a glass capillary, pulled to a fine tip, usually 1 to 2 μm in diameter, and heat polished.

permeability a characteristic of the channel pore that describes which ions are able to travel through the channel, channels are "permeable" while ions or solutes are "permeant" (see also **selectivity**).

pipette solution the solution used to fill the pipette and that equilibrates with the cytosol in whole-cell mode.

rectification nonlinear current-voltage relation indicating that the channel gating or conductance is influenced by voltage; the opposite is "Ohmic", referring to Ohm's law and describing a linear current-voltage relation in which resistance is not voltage dependent.

reversal potential the membrane potential at which there is no net current across the membrane, used to determine ionic selectivity, also called the zero-current potential.

run down a time-dependent decrease in currents often attributed to the dilution of soluble factors in the cytosol during equilibration with the pipette solution; also called **wash out**.

selectivity describes the affinity of the channel pore for different ions. Selectivity is usually determined by reversal potential and is not necessarily the same as permeability.

symmetrical solutions an experimental condition in which the bath and pipette solutions contain equal concentrations of the major permeant ions, as in "symmetrical 100 mM KCl."

tail currents currents measured at deactivating membrane potentials before the channels have had sufficient time to deactivate, useful for measuring reversal potentials in whole-cell mode because the background leak currents are effectively subtracted.

voltage dependence a description for any channel characteristic, such as gating, inactivation, or single-channel conductance, that changes with membrane potential.

whole-cell mode the physical configuration in which the pipette solution is continuous with the cell cytosol and measurements are made across the entire plasma membrane.

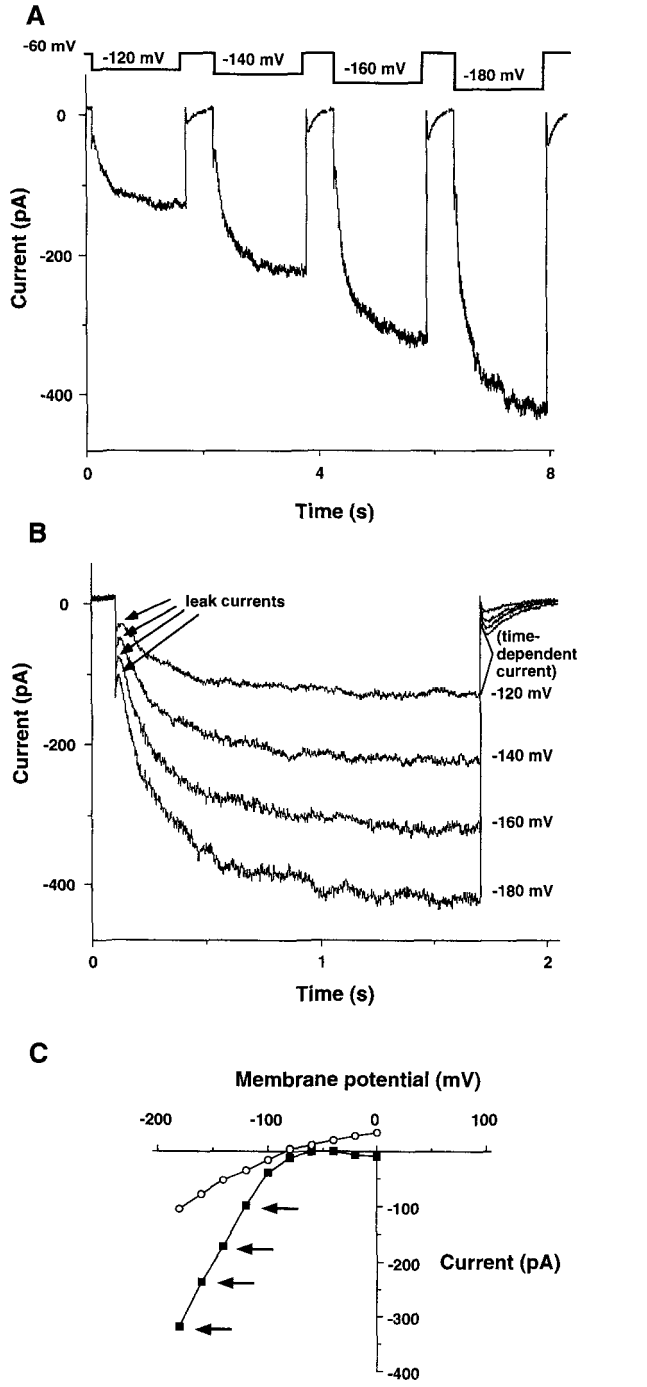

Figure 1. An example of whole-cell currents. From a holding potential of −60 mV the membrane potential was sequentially changed to more negative potentials. A, Time-dependent inward currents elicited by hyperpolarizing membrane potentials. The membrane potential is returned to the holding potential of −60 mV between test potentials. B, Typical presentation of whole-cell currents. The same data in A are superimposed. The arrows indicate the extent of leak current and the bracket on the right indicates the time-dependent current at a membrane potential of −120 mV. C, Current-voltage relation for the data in A and B. The leak current illustrated in B is plotted against membrane potential (○). The time-dependent current is plotted against the membrane potential (■). The arrows indicate data extracted from B.

leak current is plotted in Figure 1C in the form of a current-voltage relation, also called an I/V curve (the current level indicated by the arrows in Fig. 1B is plotted against the membrane potential), and has some characteristics worth mentioning.

The leak current is nearly linear, which means that the current is not voltage dependent. It is shifted to the left, indicating that part of this current is likely to be due to H^+-ATPase, which produces an outward current. Because the channel of interest in Figure 1 is time and voltage dependent, the leak current can be subtracted from the total current and the specific time-dependent currents (indicated in Fig. 1B) can be plotted on the current-voltage relation in Figure 1C. From this current-voltage relation we can determine that the currents are highly voltage dependent: the relation is nonlinear. We can also see that at potentials more positive than −60 mV the channels appear to be off (deactivated). The final important observation that we can make from these data is that this current is likely to be activated at physiological membrane potentials in the range of −100 to −150 mV. Analysis of whole-cell currents using cells from wild-type plants does not reveal whether a discrete transporter protein or a class of related proteins is responsible for the observed currents. Such a determination can only be made using mutant or transgenic (antisense or overexpressing) plants.

From the data shown in Figure 1 it is impossible to determine which ions are responsible for the time-dependent current. As stated previously, a downward current is by convention an inward current. However, this could be due to cation movement into the cell, anion movement out of the cell, or both. The ionic selectivity of a channel pore (the ability of ions to enter the pore) is one of the key characteristics determining its possible physiological role. The selectivity of an ion channel can be determined by an accurate measurement of current reversal potentials.

At the whole-cell level, current reversal potentials can be measured using a method called tail-current analysis (for example, see Schroeder et al. [1987]). This method is useful when studying time- and voltage-dependent ion channels. An example of tail-current analysis is presented in Figure 2. First, the currents were activated (in this case by a membrane potential of −100 mV), and then the membrane potential was changed to various potentials where the currents deactivate and the current records are typically superimposed, as in Figure 2A. One important feature of this type of analysis is that before the currents deactivate they can be reversed (e.g. at +100 mV; Fig. 2A) and can be measured.

The currents were measured immediately after the deactivating potential begins (left arrow of Fig. 2A), and again at steady state. This steady-state current (right arrow of Fig. 2A) represents the residual or leak current and was subtracted from the first measurement. The tail current was plotted against the voltage and, although this part of the analysis is normally not presented, this plot is shown in Figure 2B. The important information that can be derived is the reversal potential, or zero-current potential. In this case the zero-current potential is 0 mV. The experiment of Figure 2 was performed with an equal concentration of KCl on each side of the membrane (symmetrical solutions), so the Nernst potentials for K^+ and for Cl^- are both zero. The Nernst equation relates membrane potential to transmembrane concentration gradients (Hille, 1992):

$$E_{rev} = \frac{2.3\,RT}{mF} \log \frac{[X]_o}{[X]_i}$$

where E_{rev} is the membrane potential, F is Faraday's constant, R is the gas constant, and m is the valence of ion X. The ratio $[X]_o/[X]_i$ represents the concentration gradient of ion X across the membrane. To determine whether K^+ or Cl^- is permeating the channel, additional experiments using a gradient of KCl could be conducted. When considering monovalent cations, $2.3\,RT/mF$ is approximately 59 at 22°C:

$$E_{rev} = 59 \log \frac{[X]_o}{[X]_i}$$

Therefore, if a 10-fold KCl gradient were applied with a higher cytosolic concentration of KCl, the Nernst potential for K^+ would be −59 mV and that for Cl^- would be +59 mV (Eq. 2). The tail-current analysis could be repeated and any shift in the reversal potential toward the Nernst potential for either K^+ or Cl^- could be determined. Ion-selectivity studies are much easier and more accurate when performed at the single channel level, as discussed below. However, it is often difficult to correlate activities seen at the whole-cell level with those at the single-channel level. Another problem could be that single channels run down quickly or are too small to be resolved. In those cases tail-current analysis provides a good option to determine ion selectivity.

It should be noted that many currents are not voltage dependent; they are activated by factors other than voltage, such as hormones, second messengers, or increased cytosolic Ca^{2+} and, therefore, tail-current analysis is not possible. In that case, reversal potentials derived from whole-cell currents would not be specific for a single type of transporter under investigation due to the contribution of leak currents (as shown in Fig. 1). The patch-clamp technique provides several advantages for studies at the whole-cell level, as demonstrated in Figures 1 and 2: (a) The high-resistance seal between pipette and membrane makes low-noise recordings with limited contributions from leak currents possible; (b) the ability to control the ionic composition of the cytosol allows for measurement of the ion-selectivity of the transport protein; and (c) measurement of currents across the entire cell membrane provides information on transporter density in the membrane that is directly related to physiological function.

Analysis of Single-Channel Currents

All three classes of transporter proteins, pumps, cotransporters, and ion channels, have been assayed by the patch-clamp technique at the whole-cell level (for review, see Hedrich and Schroeder [1989]). In general, only ion channels can be measured in an excised patch of membrane

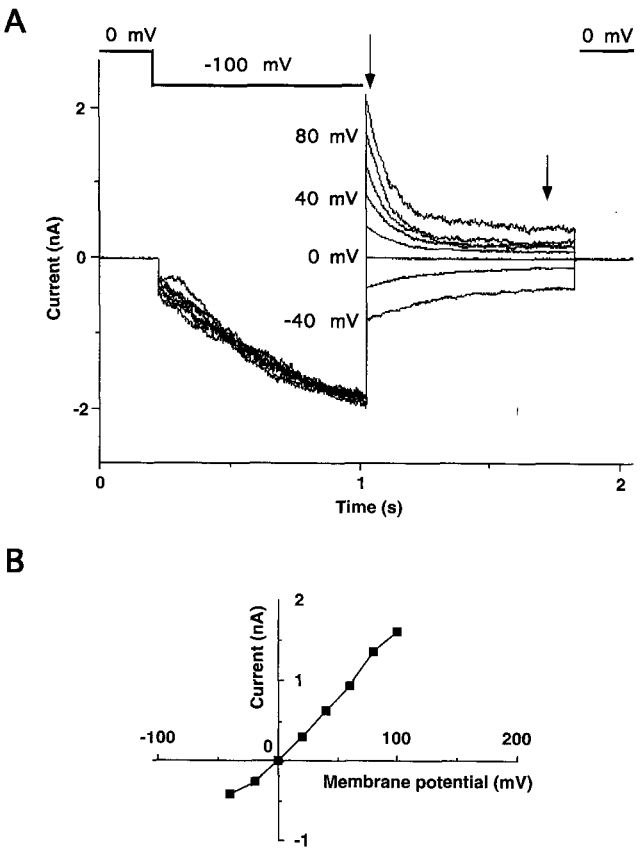

Figure 2. An example of tail-current analysis. A, Inward currents were activated by hyperpolarizing membrane potential of −100 mV, then the membrane potential was shifted to progressively less negative potentials. The data were then superimposed. B, Currents were measured at times indicated by arrows in A. The steady-state currents (right arrow; see text) were subtracted and the remaining tail currents were plotted against the membrane potential to determine a current reversal potential.

because pumps (and probably also cotransporters) only transport one or a few ions per transport cycle, which requires a conformational change in the transport protein. The result is that the rate of transport is usually less than 1×10^3 per second, or about 1000-fold lower than the resolution limit for single-channel recording. The maximum rate for ion channels is limited by diffusion, because once ion channels are open, a protein conformational change is not required for transport. However, conformational changes in ion-channel proteins are extremely important for single-channel recordings. The change from open to closed, evident as the typical "flickering" current, is a conformational change allowing a single-channel protein to be identified by its characteristic conductance. Without this fortuitous feature it would be difficult to resolve ion channels from the background leak currents.

The giga seal between the glass pipette and the cell membrane, in addition to providing high electrical resistance, is also physically strong and allows a patch of membrane to be removed from a cell (Hamill et al., 1981; Schroeder et al., 1984; Hille, 1992). The result is a configuration with a small patch of membrane sealed to the pipette with the bath solution on one side and the pipette solution on the other. In this configuration it is possible to record currents derived from ion flux through single ion-channel proteins. As the channel pore opens and closes, the currents turn on and off. Therefore, it is possible to measure conformational changes in single-transport proteins with this method, and the patch-clamp technique is truly a molecular tool for electrophysiologists.

The resolution of a patch-clamp setup is limited by the noise generated by the amplifier. The noise level from a modern amplifier is around 0.2 pA and, therefore, the smallest discrete current transition that can easily be resolved without noise analysis is also around 0.2 pA. This is the amount of current through a 2-pS conductance at 100 mV from the zero current potential. Another way of looking at the situation is that 0.2 pA is about 1.2×10^6 monovalent ions per second moving through a single pore. Conductances of 1 to 150 pS are typical for ion channels in plants and other organisms. It is also reasonable to expect that ion channels with conductances smaller than 1 pS may be predominant but more difficult to measure experimentally because of the limits of resolution. Other methods such as noise analysis are required to detect channel activity when conductances are small (Tyerman et al., 1995)

An example of single-channel currents is presented in Figure 3A. In this experiment the membrane potential was held at 0 mV, shifted to other potentials for 1.8 s, and then returned to the holding potential. In the top trace the membrane potential was shifted from the 0-mV holding potential to 80 mV and back to 0 mV. There are several important observations that can be made from these data. First, at the 0-mV holding potential, ion-channel openings were apparent. This is expected under conditions in which a selective channel is present and different solutions are used on either side of the membrane. Therefore, the ion channels are activated at both 0 and 80 mV. From this experiment we cannot make any observations concerning the kinetics of activation. Second, the flickering currents were caused by a single ion-channel protein with a large conductance in the membrane patch. The current amplitude between the open and closed states can be measured (indicated on the right in Fig. 3A) and plotted against the membrane potential.

The leak current is approximately the current present when the channel is closed; it has several possible components, such as smaller ion channels, pump and carrier currents, and ions leaking at the pipette/membrane seal. When current due to single-channel openings is measured, the leak currents are effectively subtracted. Analysis of the data shown in Figure 3A reveals the single-channel conductance and current reversal potential, and shows whether the currents are rectified. The single-channel conductance is provided by the slope of the current-voltage relation shown in Figure 3C, which is roughly 0.05 pA mV^{-1} or about 50 pS, an average-sized ion channel. The current reversal potential, also called the zero current potential, is approximately −40 mV and in this case must be extrapolated.

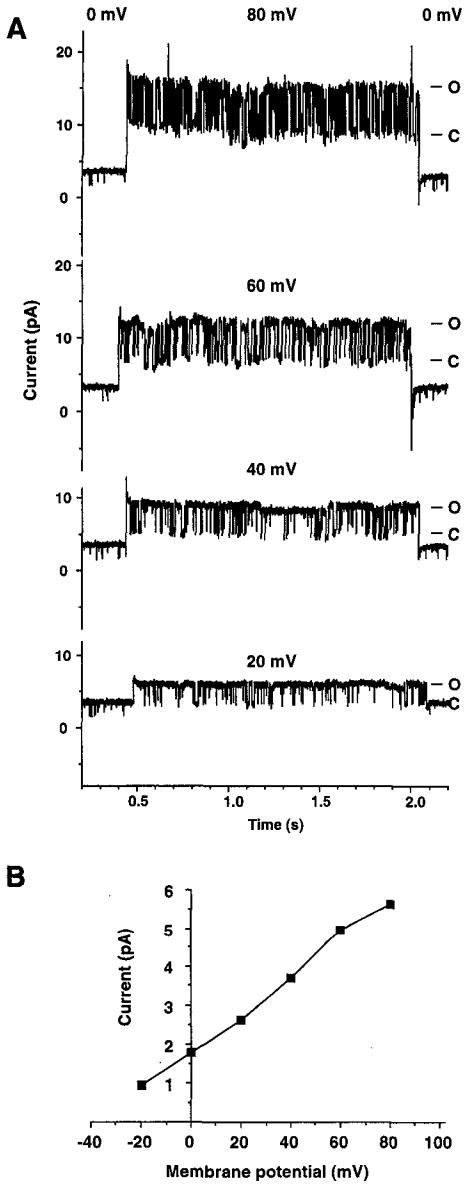

Figure 3. An example of analysis of single-channel currents. A, From a holding potential of 0 mV the membrane potential was changed to 80, 60, 40, and 20 mV for a period of 1.8 s. O and C, The open and closed states for the ion channel, respectively. B, Current-voltage relation for the data in A. The major single-channel currents were measured and plotted against the membrane potential to determine the current reversal potential.

To collect ion-selectivity data, experiments such as this are often performed using biionic conditions, in which two ions being compared are present on opposite sides of the membrane; for example, equal concentrations of KCl in the pipette and CsCl in the bath. This was the case for the experiment in Figure 3. If the reversal potential is around 0 mV, then the two ions enter the channel pore equally well. If the reversal potential is shifted, as in the example, then one ion enters the channel pore more easily and the channel is said to be selective for that ion. The relationship between reversal potential and relative ion selectivity is given by the Nernst equation (Hille, 1992). It should be noted that selectivity data based on reversal potentials does not describe whether an ion necessarily traverses the pore; in fact, ion channels are usually selective for ions that block conductance. Other types of experiments are necessary to determine which ions are actually permeant. The current-voltage relationship shown in Figure 3B is approximately linear, indicating that under these conditions the single-channel conductance was not voltage dependent.

The high-resolution data provided at the single-channel level can help to explain some of the observations made at the whole-cell level. For example, if we know the single-channel conductance, we can calculate the number of ion channels responsible for activity measured in whole cells. By determining the open probability (the probability that a channel will be in the open state) at various membrane potentials, it is often possible to explain voltage-dependent characteristics observed at the whole-cell level (as in Figs. 1 and 2). However, in many cases it is difficult to associate activities observed at the whole-cell level with single channels observed in patches. This is because many currents are due to channels or cotransporters that are too small to resolve (<1 pS) or the ion channels may run down too quickly for single-channel measurements (channel activity may require cytosolic factors that are lost when a patch of membrane is isolated) to be made.

MOLECULAR BIOLOGY FOR PLASMA MEMBRANE TRANSPORTERS

Expression Cloning and Heterologous Expression

A biochemical approach to obtaining genes for plasma membrane transporters has only been successful for H^+-ATPase (for review, see Sussman [1994]), which is relatively abundant in the plasma membrane (compared with other transporters) and retains an enzymatic activity that can be assayed in solution during purification. Ion channels and cotransporters do not offer either of these advantages and, therefore, biochemical approaches to clone their cDNAs have failed. Screening cDNA libraries using heterologous probes for transporters has similarly met with little success. However, the cloning of AKT2/AKT3 using a conserved sequence of the K^+-channel H5 pore domain is an example in which this approach was successful (Ketchum and Slayman, 1996).

The most powerful technique for isolating plasma membrane transporters has been expression cloning using the yeast *Saccharomyces cerevisiae*. The first Suc transporter, SUT1, was cloned from spinach using this technique (Riesmeier et al., 1992). That work illustrates how appropriate yeast strains can be constructed to clone specific transporters. Yeast does not have Suc transporters in the plasma membrane, but can utilize Suc as a carbon source because it has extracellular invertase activity. Therefore, to clone the cDNA of a Suc transporter, an invertase knockout was utilized that expressed Suc synthase in the cytosol. The resulting strain failed to grow on Suc unless a plasma membrane Suc transporter was simultaneously expressed. Within the last several years, the first cDNAs for plant K^+

channels (Anderson et al., 1992; Sentenac et al., 1992), amino acid transporters (Frommer et al., 1993, 1995; Hsu et al., 1993), NH_4^+ transporters (Ninnemann et al., 1994), high-affinity K^+ transporters (Schachtman and Schroeder, 1994), SO_4^{2-} transporters (Smith et al., 1995), oligopeptide transporters (Rentsch et al., 1995), and Pro transporters (Rentsch et al., 1996) have been isolated using functional expression in yeast. Following the completion of the yeast sequence a comprehensive evaluation of transporter genes was made (Andre, 1995). A function has not yet been assigned for many of the yeast transporters; however, we can expect that once the newly identified genes are mutagenized and functions for the transporters are obtained, many additional yeast strains will be useful for functional cloning experiments.

The utility of functional expression of plant transporters in yeast extends far beyond the isolation of novel genes. Randomly mutated plant transport genes can be selected for altered function in yeast. For example, this strategy has been used to isolate Glc transporters from *Chlorella kessleri* with altered K_m (Will et al., 1994) and K^+ channels with drastically altered ionic selectivity (Uozumi et al., 1995), and to generate Na^+-coupled high-affinity K^+ transporters with a greater K^+/Na^+ selectivity (Rubio et al., 1995). In principle, this approach can be used to engineer changes in substrate specificity for any cloned plasma membrane transporter.

Clues to the transport mechanism of genes cloned by functional expression in yeast can be obtained by sequence comparisons and by assays of radioactive substrate uptake by yeast; however, high-resolution assays are necessary to definitively determine transport mechanisms. Direct patch-clamp analysis of yeast expressing cloned plant transporters is an attractive approach (Bertl et al., 1995), but is difficult because of the small size of yeast cells. The expression system utilized most extensively for this purpose is *X. laevis* oocytes (for a recent review, see Schroeder [1994]). In this approach, cRNA transcripts are injected into oocytes that provide a low background and very active translational activity. Many plant transporters, initially cloned by functional complementation in yeast, have been characterized in detail using oocyte expression. Examples include the K^+ channel KAT1 (Schachtman et al., 1992), the high-affinity, Na^+-coupled K^+ transporter HKT1 (Rubio et al., 1995; Gassmann et al., 1996), the amino acid transporter AAP1 (Boorer et al., 1996a), and the Suc transporter SUT1 (Boorer et al., 1996b).

Genetics: Forward and Reverse

Ion and solute transport across the plasma membrane are considered to be important for basic physiological processes such as growth and development, and for responses to hormones and environmental signals. Therefore, it is somewhat surprising that traditional genetic approaches have generally not been successful in obtaining transport protein genes. An exception is the cloning of the NO_3^- transporter gene CHL1 (Tsay et al., 1993). Arabidopsis plants with T-DNA insertional mutations were selected by their ability to grow in the presence of toxic concentrations of ClO_3^-, an analog of NO_3^-. There are two likely reasons that genetic approaches have not been successful for the identification of other transporter genes. First, mutations in transport protein genes may be lethal. Evidence for this situation thus far is indirect; for example, antisense inhibition of the Suc transporter SUT1 in potato results in an extreme phenotype, indicating that the null mutation is likely to be lethal (Riesmeier et al., 1994). Second, transport protein genes may be redundant. Considering the large multigene families of H^+-ATPases in plants (Sussman, 1994), it is likely that some may have overlapping expression and activity.

Recent advances in the reverse-genetic approach will overcome the problems associated with both lethal mutations and redundant genes. This strategy involves identifying Arabidopsis T-DNA insertional mutants using PCR (McKinney et al., 1995; Krysan et al., 1996). In this method primer pairs corresponding to the gene of interest and T-DNA border regions are used to detect tagged genes in pools of DNA from tagged mutants. Heterozygous tagged mutants can be identified and it is straightforward to self-fertilize these plants to determine if the homozygous tagged plants survive or not. Therefore, a knockout producing a lethal phenotype can be identified and studied. In the case of redundant gene function, in which null mutants display no phenotype, homozygous tagged plants can be obtained. By making crosses, plants with disruptions in multiple genes can be obtained and identified using PCR and the physiological consequences can be determined.

An extremely useful outcome of this technology is that knockout-replacement approaches will be possible. Assays in a null background of the effects of mutant genes, ectopically expressed genes, and genes from other plants or other organisms will be possible. Even in the case in which a gene knockout results in a lethal phenotype, a plant carrying the replacement gene can be crossed with the heterozygous knockout plant. The resulting F_1 plants can be self-fertilized and the homozygous knockout-replacement candidates can be selected by PCR. Using this system, different mutants, perhaps those selected by yeast functional expression, can be tested for their ability to complement lethal mutations in plants.

Electrophysiological Assays of Mutant and Transgenic Plants

Identifying the regulatory mechanisms of plasma membrane transporters is one of the most difficult problems facing electrophysiologists, because interactions with other proteins are often directly or indirectly involved. Although expression in oocytes offers a good system to assay isolated plant transporters, studies of physiological regulation need to be performed in plants. Recently, electrophysiological assays have been applied to signal transduction mutants in Arabidopsis. Experiments performed using guard cells provide an example of this approach. The response of guard cells to ABA involves the regulation of K^+ and anion channels in the plasma membrane (for review, see Ward et al. [1995]). Mutations in the *abi1* and *abi2* loci in Arabidopsis cause an insensitivity to ABA. The *abi1* gene encodes a

protein phosphatase and a mutated form of the *abi1* gene was recently expressed in tobacco (Armstrong et al., 1995). Electrophysiological analysis of the K^+ channels in this tobacco line revealed that ABA-induced regulation of inward and outward K^+ channels was reduced. The patch-clamp technique was recently applied to Arabidopsis guard cells from wild-type, *abi1–1*, and *abi2–1* plants (Pei et al., 1997). ABA was shown to activate anion channels in wild-type Arabidopsis guard cells, but not in *abi1–1* or *abi2–1* guard cells. This represents the first example in which mutations are known to affect signal transduction between hormone receptors and ion channel regulation. Additional work is required to determine if the *abi1* or *abi2* gene products participate in the signaling pathway in wild-type plants. However, suppresser mutations will certainly affect signaling components and may identify proteins that interact directly with the guard cell anion channels.

FUTURE PROSPECTS

Many plasma membrane transporters have now been cloned, mostly using yeast complementation, and basic information on the transport mechanism for most of these proteins has been obtained, in many cases using oocyte expression. However, for almost all plasma membrane transporters the most physiologically important information is lacking: expression pattern, mechanism of regulation of protein level, regulation of transport activity, and interaction with other proteins. Many of these problems will require direct assay of transporters in plants. For example, knockout-replacement studies now offer a means to determine if mutations in potential regulatory sites affect transporter function. The expression of reporter-tagged transporter proteins in transgenic plants will provide a way to identify protoplasts derived from specific types of cells to extend the utility of the patch-clamp technique. Direct patch-clamp analysis using plants ectopically expressing mutant transporters or mutated regulatory proteins, as well as further patch-clamp analysis of signal transduction mutants, will provide insight into the roles of transporters in physiological responses.

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